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An Activating $G_s\alpha$ Mutation Is Present in Fibrous Dysplasia of Bone in the McCune-Albright Syndrome

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ABSTRACT

McCune-Albright syndrome (MAS) is a sporadic disease characterized by polyostotic fibrous dysplasia, café-au-lait spots, and multiple endocrinopathies. The etiology of fibrous dysplasia is unknown. Activating mutations of codon 201 in the gene encoding the α-subunit of G_s, the G-protein that stimulates adenylyl cyclase, have been found in all affected MAS tissues that have been studied. Initial attempts to amplify DNA from decalcified paraffin-embedded bone specimens from MAS patients were unsuccessful. Therefore, we analyzed DNA from frozen surgical bone specimens from five MAS patients using polymerase chain reaction and allele-specific oligonucleotide hybridization. Most of the cells in four specimens of dysplastic bone contained a heterozygous mutation encoding substitution of Arg^{201} of $G_s\alpha$ with His, but the mutation was barely detectable in peripheral blood specimens from the patients. Only a small amount of mutant allele was detected in a specimen of normal cortical bone from the fifth patient, although this patient had a high proportion of mutation in other, affected tissues. The mosaic distribution of mutant alleles is consistent with an embryological somatic cell mutation of the $G_s\alpha$ gene in MAS. The presence of an activating mutation of $G_s\alpha$ in osteoblastic progenitor cells may cause them to exhibit increased proliferation and abnormal differentiation, thereby producing the lesions of fibrous dysplasia. (*J Clin Endocrinol Metab* **79:** 750–755, 1994)

McCUNE-ALBRIGHT syndrome (MAS) is a sporadic disease classically defined by polyostotic fibrous dysplasia, café-au-lait spots, sexual precocity, and other hyperfunctional endocrinopathies (1). An activating missense mutation in the gene for the α-subunit of G_s, the G-protein that stimulates cAMP formation, has been discovered in these patients (2–4). The mutation is found in variable abundance in different affected endocrine and nonendocrine tissues, consistent with the mosaic distribution of abnormal cells generated by a somatic cell mutation early in embryogenesis (5). Substitution of Arg^{201} with either Cys or His inhibits the GTPase activity of $G_s\alpha$ and leads to inappropriate stimulation of adenylyl cyclase (6).

Fibrous dysplasia can occur in monostotic and polyostotic forms, with or without other features of MAS. Dysplastic lesions are characterized by the progressive replacement of the normal medullary architecture of bone with abnormal fibroosseous tissue (1, 7–16). These expanding lesions often cause pathological fractures, deformity, pain, and nerve compression. Lesions are composed of spindle-shaped fibroblastic cells and minimal extracellular matrix in a swirled pattern, surrounding trabeculae of immature metaplastic woven bone. Irregular islands of cartilage are sometimes present. The identity of the predominant fibroblastic cell is unknown, but it is presumably a member of the osteoblast lineage. Although osteoblastic activity is present in the lesions, the immature bone spicules are never converted into normal lamellar bone.

Nonsurgical treatment of fibrous dysplasia is not effective, and radiotherapy can increase the risk of malignant transformation (1, 13, 14). Although surgical treatment is available for some of the complications of fibrous dysplasia (10, 12–14), many patients, nevertheless, suffer significant disability and deformity. Deformity of the chest wall and arteriovenous shunting through dysplastic bone may contribute to cardiopulmonary insufficiency in some MAS patients (1, 13). A better understanding of the molecular pathophysiology of fibrous dysplasia might reveal new avenues for medical therapy.

We suspected that the same Arg^{201} $G_s\alpha$ mutation found in abnormal endocrine and nonendocrine tissues of MAS patients was also responsible for the associated lesions of polyostotic fibrous dysplasia, but initial attempts to amplify DNA from paraffin-embedded MAS bone samples were unsuccessful (2). Therefore, we prospectively analyzed DNA samples prepared from surgical bone specimens from MAS patients. A preliminary account of this work has been published in abstract form (17).

Subjects and Methods

Analysis of archival paraffin-embedded bone samples

Crude DNA was isolated from slides of unstained paraffin-embedded bone, as previously described for other tissues (2). Adjacent slides stained with hematoxylin and eosin were used to identify regions of interest. Nine dysplastic bone samples (from six MAS patients) and one normal

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bone specimen were analyzed. Standard primers (PC03 and PC04) that amplify a 110-basepair fragment of the human β-globin gene were used for polymerase chain reaction (PCR), as previously described (18). Assay components were heated to 80 C in the thermal cycler (Perkin-Elmer/Cetus, Norwalk, CT) before adding *Taq* polymerase. PCR products were resolved on a polyacrylamide gel and visualized with ethidium bromide.

Analysis of DNA from frozen bone samples, blood, and other tissue

Five samples of bone obtained at the time of surgery were frozen and stored at −70 C. Frozen bone was partially defrosted, inspected, and dissected into small pieces. Genomic DNA was extracted from a section directly adjacent to that used for histological studies. Each bone section for DNA extraction (~5 mm) was placed in a microfuge tube, washed twice with PBS, and pulverized. Digestion buffer [50 mmol/L Tris (pH 8.5), 1 mmol/L EDTA, 0.5% Tween-20, and 0.2 mg/mL proteinase-K] was added (0.2–0.7 mL/tube), and samples were incubated at 37 C overnight or at 56 C for 1 h. Samples were centrifuged to remove undigested debris, incubated at 95 C for 8 min, and then stored at 4 C. DNA in 5 μL of the supernatant from each sample was used as the template for PCR. Genomic DNA from blood and other paraffin-embedded tissue was prepared as previously described (2).

A short fragment of the $G_s\alpha$ gene (exon 8) containing the Arg^{201} codon was amplified with PCR, and mutations were identified with allele-specific oligonucleotide hybridization, as previously described (2). This technique measures the specific binding of short, radioactively labeled oligonucleotide probes that either match the wild-type DNA sequence exactly (R201) or contain a single base substitution encoding His (R201H) or Cys (R201C). DNA isolated from paraffin-embedded normal adrenal tissue was used as a negative control, and DNA from pituitary tumors with known Arg^{201} mutations was used as a positive control (19).

Patients

Patient 1 was a 28-yr-old female with café-au-lait pigmentation, sexual precocity, hyperthyroidism, and polyostotic fibrous dysplasia involving the skull, maxilla, mandible, vertebrae, and femurs. A specimen of dysplastic bone was obtained during left maxillary curettage performed because of facial pain and deformity.

Patient 2 was a 7-yr-old female with café-au-lait pigmentation, sexual precocity, hyperthyroidism, and polyostotic fibrous dysplasia involving the skull, vertebrae, right forearm, left foot, and right femur. This patient was previously reported as patient 1 (20) and patient 7 (21). A specimen of dysplastic bone was obtained during an orthopedic procedure to repair a right femoral fracture.

Patient 3 was a 7-yr-old female with café-au-lait pigmentation, sexual precocity, mild hyperthyroidism, and polyostotic fibrous dysplasia involving the skull, both arms, and both legs. At age 8 months, the patient underwent right oophorectomy and biopsy of a left ovarian cyst. This patient was previously reported as patient 8 (20) and patient 11 (21). A specimen of dysplastic bone was obtained during a therapeutic orthopedic procedure that included right femoral osteotomy.

Patient 4 was a 5-yr-old boy with café-au-lait pigmentation and polyostotic fibrous dysplasia involving the skull, a rib, the right humerus, right fibula, and both femurs. The patient had an episode of acidosis during infancy that required therapy with sodium bicarbonate, a finding that has been reported in several other infants with MAS (4). A specimen of dysplastic bone was obtained during right intramedullary femoral rod insertion.

Patient 5 was a 33-yr-old female with café-au-lait pigmentation, sexual precocity, hyperthyroidism, acromegaly, left intraductal breast carcinoma, and polyostotic fibrous dysplasia involving the skull, facial bones, femurs, pelvis, and vertebrae. Bone biopsy of an unaffected area in the iliac crest was obtained while the patient was undergoing placement of a left intramedullary femoral rod. A sample of dysplastic bone was not obtained during the procedure.

Results

Previous attempts to amplify a small DNA fragment encompassing exon 8 of the $G_s\alpha$ gene from several samples of paraffin-embedded MAS bone were unsuccessful (2). To confirm our suspicion that the bone samples had been decalcified in acidic solutions and did not contain any DNA template suitable for PCR, we attempted to amplify an even smaller target DNA fragment from the human β-globin gene using standardized conditions (18). Paraffin-embedded samples of testes and kidney provided adequate DNA templates for this assay, but no PCR products were obtained from 10 different paraffin-embedded bone samples (data not shown).

All frozen undecalcified bone samples yielded DNA adequate for PCR amplification. In patient 1, the R201H mutant allele was present in high proportion in a specimen showing irregular woven bone formation within a cellular fibroproliferative background, but was barely detectable in the peripheral blood (Figs. 1A and 2). In patient 2, the R201H mutation was abundant in a bone specimen containing abnormal cartilage with endochondral ossification and microscopic foci of more typical appearing fibrous dysplasia, but was not present in peripheral blood (Figs. 1B and 2). In patient 3, the R201H mutation was present in a specimen consisting entirely of cellular fibrous tissue and woven bone spicules, but was barely detectable in the peripheral blood (Fig. 2). The R201H mutation was also detected in surgical samples of right ovary and a vaginal cyst from patient 3 (data not shown).

Much of the specimen from patient 4 contained shards of lamellar bone and cartilage. It was unclear whether the cartilage represented normal growth plate or was lesional. In addition, there was an area containing several moderately cellular woven bone trabeculae within fairly dense fibrous tissue, consistent with fibrous dysplasia. This specimen contained a significant proportion of mutant allele (Fig. 2). Another specimen from patient 4 containing a mixture of normal and abnormal components had a slightly higher proportion of mutant allele (data not shown). The fact that the ratio of mutant allele to wild-type allele is lower than that found in specimens from the first three patients may be attributed to the heterogeneous nature of the specimens from patient 4.

An area of bone unaffected by fibrous dysplasia was biopsied in patient 5. Analysis of sections, including special stains, fluorescent microscopy, and histomorphometry, revealed normal-appearing cortical and cancellous bone with the possibility of mild osteomalacia, but no fibrous dysplasia (Fig. 1C). This specimen contained only a trace of the mutant R201H allele (Fig. 2). Mutation was not detected in another specimen of normal-appearing cortical bone and capsular tissue (data not shown). No mutation was detected in blood, although the R201H mutation was found in several other affected tissues from this patient, including pituitary adenoma (Fig. 2).

Discussion

Archival paraffin-embedded bone did not serve as a template for PCR amplification, even when optimal assay con-

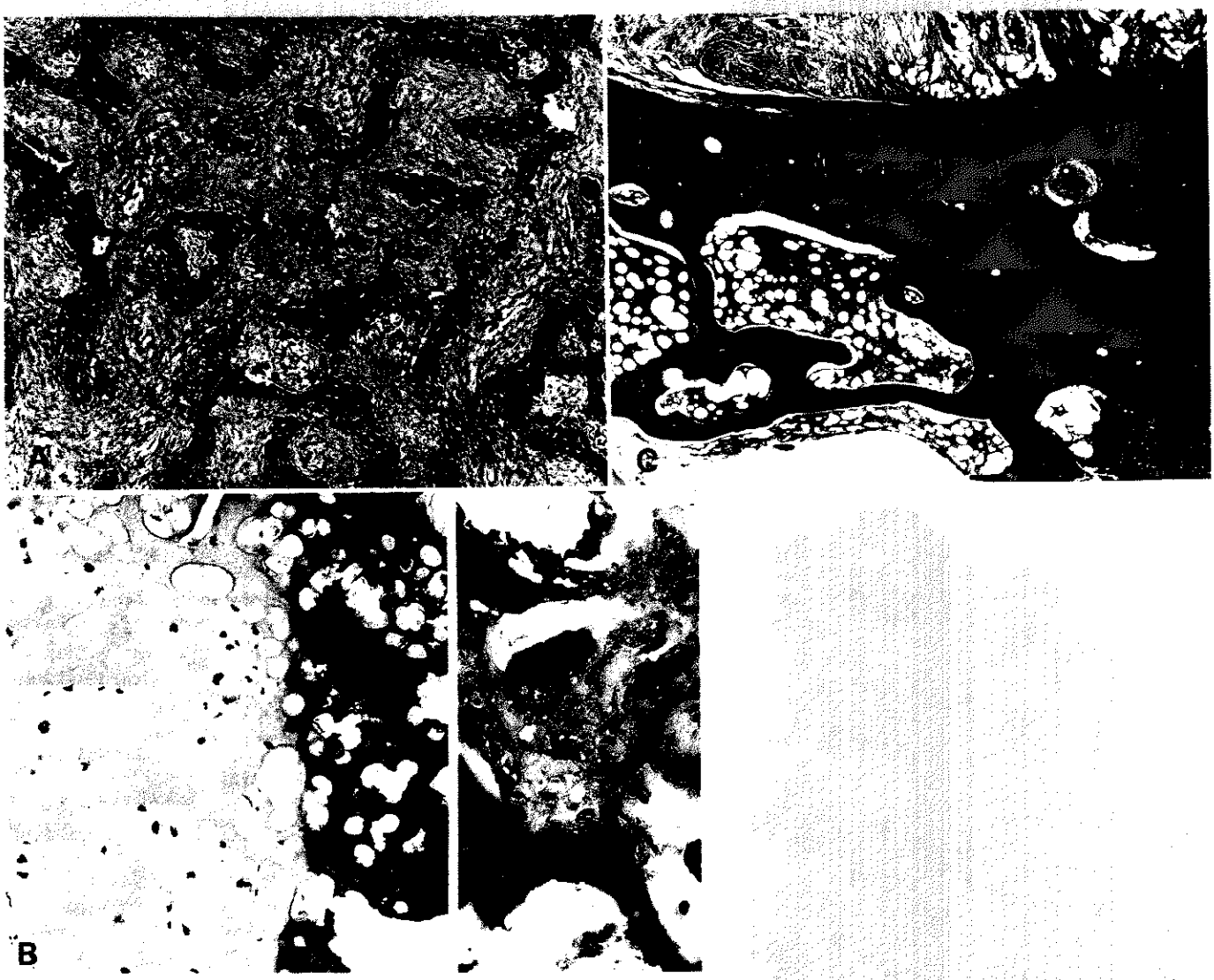

FIG. 1. Photomicrographs of hematoxylin- and eosin-stained bone sections from three MAS patients. A, The bone specimen from patient 1 (magnification, ×75) shows irregular woven bone trabeculae being formed within a cellular fibroproliferative background. The trabecular surfaces reveal modest numbers of osteoclasts and osteoblasts; the latter are more difficult to recognize because of their somewhat flattened appearance. The newly formed bony spicules follow the swirled pattern of the fibrous tissue. B, Composite photomicrograph of the bone lesion from patient 2. *Left* (magnification, ×75), Hyaline cartilaginous component maturing toward endochondral bone formation, as evidenced by the darkly stained provisional zone of calcified cartilage. *Right* (magnification, ×400), The fibrous dysplastic features of the lesion are illustrated by a fibroproliferative component and calcified woven bone ossicle. C, The specimen from patient 5 (magnification, ×30) contains normal-appearing cortical and cancellous bone and marrow, with no evidence of fibrous dysplasia.

ditions were used. This is presumably because bone specimens are routinely decalcified in acidic solutions, a procedure known to destroy DNA (18, 22), before they are embedded in paraffin. Retrospective genetic analysis of archival clinical specimens is possible only if the tissues have been properly preserved. Freezing bone specimens is one option (23), and samples decalcified with EDTA and embedded in plastic have also been shown to provide a suitable template for PCR (24). Orthopedic surgeons and pathologists should be aware of this fact when handling bone samples that may be valuable for future molecular biological studies.

Analysis of DNA from frozen bone specimens indicates that the same somatic $G_s\alpha$ gene mutation found in other affected tissues of MAS patients is also detected in lesions of fibrous dysplasia. Moreover, the equal intensity of wild-type and mutant oligonucleotide binding to DNA prepared from the entirely dysplastic bone specimens of patients 1, 2, and 3 indicates that most or all of the cells in those lesions contain a heterozygous mutation encoding substitution of Arg^{201} of $G_s\alpha$ with His. The mutation was barely detectable in peripheral blood specimens from the same patients. Only a small proportion of mutant allele was detected in a specimen of normal cortical bone from the fifth patient, although this patient had a high proportion of mutation in other affected tissues. The mosaic distribution of mutant alleles is consistent with an embryologic origin of the $G_s\alpha$ gene mutation in MAS

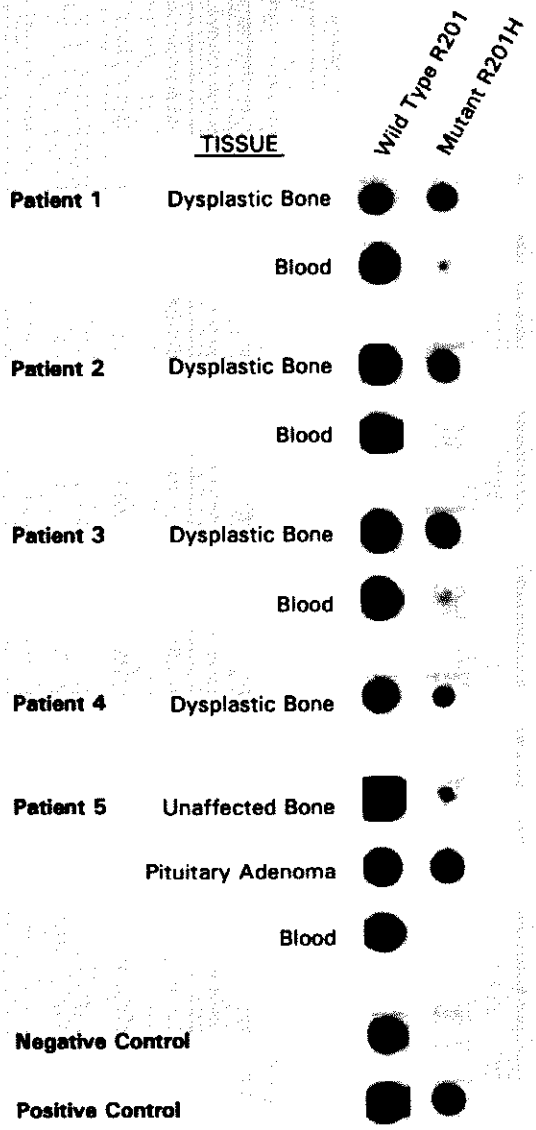

FIG. 2. Analysis of DNA samples from MAS patients. PCR-amplified genomic fragments encompassing exon 8 of $G_s\alpha$ from tissue or blood from patients and from control tissues were hybridized with wild-type R201 and mutant R201H oligonucleotide probes. There was no hybridization of DNA with the mutant R201C oligonucleotide probe (not shown).

(5). Our findings confirm those recently obtained with bone samples from one patient with an atypical presentation of MAS (25).

Although all of the patients in our study were found to have the mutation encoding substitution of Arg^{201} with His, we found the mutation encoding Arg^{201} to Cys in cells derived from dysplastic bone lesions from two other MAS patients (unpublished results) and in affected tissues from several other MAS patients with a history of polyostotic fibrous dysplasia, but from whom fresh-frozen bone samples were not available (2, 4). Somatic Arg^{201} mutations of $G_s\alpha$ are also present in cells from two cases of isolated monostotic fibrous dysplasia (26). It is possible that different molecular defects underlie other, inherited forms of fibrous dysplasia (13, 14, 16).

The process that gives rise to the distinctive lesions of fibrous dysplasia is unknown. Soon after the disease was first described, several possible etiologies were proposed (7–9). Sternberg and Joseph (7) theorized that the lesions of fibrous dysplasia in MAS reflected a secondary, massive, unsuccessful repair effort in response to a primary destructive process of unknown nature. In contrast, Lichtenstein and Jaffe (8) believed the skeletal lesions reflected "a congenital anomaly of development" and were a direct result of "the perverted activity of the specific bone-forming mesenchyme." Our finding that most or all of the abnormal fibroblastic cells seen in typical lesions of fibrous dysplasia in MAS contain a copy of the mutant $G_s\alpha$ allele lends support to the Lichtenstein and Jaffe model and suggests that the lesions result from the deviant behavior of these cells.

Exactly how persistent activation of $G_s\alpha$ might cause fibrous dysplasia is not clear. The process of normal bone formation is very complex, and the roles of cAMP production and other regulatory mechanisms are incompletely understood (27–29). We hypothesize that persistent inappropriate stimulation of G_s-mediated pathways disrupts the ordered developmental pattern normally exhibited by osteoblastic precursor cells (27–29) and causes them to undergo increased proliferation and abnormal or incomplete differentiation (15, 30).

Some endogenous factors affecting osteoblast development, such as PTH and prostaglandins, act by increasing osteoblast cAMP levels (31). Concentrations of PTH and forskolin that stimulate cAMP accumulation in cultured rat calvarial bone cells inhibit osteoprogenitor differentiation, but do not affect the cell proliferation rate (32, 33). Other cellular effects of $G_s\alpha$ activation also need to be considered. For example, activation of $G_s\alpha$ has been shown to inhibit differentiation of mouse 3T3-L1 fibroblasts, an effect that is independent of increased cAMP production (34).

Underactivity or overactivity of G_s-mediated pathways involved in bone formation may be implicated in the skeletal manifestations of two other diseases, Albright's hereditary osteodystrophy and hyperparathyroidism, respectively. Patients with Albright's hereditary osteodystrophy often have short metacarpals, phalanges, and metatarsals and exhibit ectopic ossification of soft tissue (35). These abnormalities may, in fact, reflect decreased proliferation and inappropriate differentiation of osteoprogenitor cells that are deficient in $G_s\alpha$ (36, 37). Lesions of osteitis fibrosa cystica found in patients with severe hyperparathyroidism bear a superficial resemblance to those of fibrous dysplasia, because both involve replacement of normal bone marrow elements with fibrous tissue (38). Whereas osteoclastic bone resorption is uniformly prominent in osteitis fibrosa cystica, there is a relative paucity of active osteoclasts in fibrous dysplasia, except along the advancing margins of the lesion. Although some of the actions of PTH are known to be mediated by the G_s pathway, the consequences of inappropriate PTH receptor stimulation and $G_s\alpha$ overactivity are clearly not identical in bone.

Although the predominant cell in lesions of fibrous dysplasia is likely to be an intramedullary osteoprogenitor, its exact position in the osteoblastic lineage is unknown. The proliferating cells exhibit alkaline phosphatase activity and osteocalcin immunoreactivity (39–41), two markers for the osteoblast lineage. Normally, the expression of these markers is inversely related to cell growth, but dysregulated patterns of gene expression are known to occur in transformed osteoblast and osteosarcoma cell lines (27, 29). Osteoblast-like cells can be cultured from lesions of fibrous dysplasia (30, 41), and preliminary results indicate that these cells have an increased proliferative rate and an "immature" phenotype compared to cells cultured from normal bone (30). Measuring the specific pattern of bone matrix proteins, growth factors, hormone receptors, and other antigens (29, 40–43) that are expressed by the abnormal cells in fibrous dysplasia may provide insight into the deranged differentiation process associated with the presence of mutant $G_s\alpha$.

Identifying the nature of the abnormal fibroblastic cells *in vivo* and characterizing their behavior in culture may also be helpful in the development of rational medical therapy for fibrous dysplasia. For example, drugs that decrease intracellular cAMP production in dysplastic cells might counteract some effects of the activating $G_s\alpha$ mutation. Agents that mimic or inhibit the effects of the various growth factors, cytokines, and hormones that normally regulate bone cell proliferation and differentiation may provide additional therapeutic options in the future.

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